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USE OF A VACCINIA CONSTRUCT EXPRESSING THE
CIRCUMSPOROZOITE PROTEIN IN THE ANALYSIS OF PROTECTIVE
IMMUNITY TO PLASMODIUM YOELII

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ABSTRACT Antibodies to the circumsporozoite (CS)
protein may play a role in the protective immunity
induced by immunization with irradiated sporozoites.
However, the observation that protection against a large
(5000 sporozoites) inoculum is dependent on T cells of
the suppressor/cytotoxic phenotype (CD8) has clearly
established the importance of cellular mechanisms in
this model system. In an attempt to stimulate a
protective cellular immune response, Balb/CByJ mice were
immunized intraperitoneally with one to 4 doses of a
vaccinia recombinant construct expressing the entire CS
protein of Plasmodium yoelii. The mice were challenged
2 weeks after the last dose of vaccine with 200 or
10,000 sporozoites. Mice vaccinated with irradiated

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sporozoites were protected, but all of the animals
immunized with the recombinant construct became
infected, even though the anti-sporozoite IFA titers of
both groups of vaccinated mice were comparable. To
determine if antibodies alone might be responsible for
protecting mice immunized with irradiated sporozoites
against a low dose challenge, we depleted them of CD8+ T
cells, and found that they were no longer protected
against even a 200 sporozoite challenge . It is unclear
whether failure to protect mice with the vaccinia
CS construct results from antigen presention inadequate
to induce the required cell mediated immune response, or
whether the CS protein is not the appropriate antigen.
It does appear however, that antibodies induced by the
irradiated sporozoite vaccine are not an adequate
explanation for the protection observed against even a
low dose challenge.

INTRODUCTION

Malaria is transmitted to humans by anopheline
mosquitoes that inject sporozoites while feeding.
Initial attempts to develop subunit sporozoite vaccines
focused on developing immunogens that induced high
levels of antibodies to the repeat regions of the
circumsporozoite (CS) protein of the sporozoite. This
approach has thus far proved only marginally successful
in humans (1,2). Furthermore, synthetic peptides and
E. coli produced recombinant vaccines have been shown
to induce protection against moderate (500-1000), but
not large (10,000) sporozoite challenge in the
Plasmodium berghei rodent model (3,4), but no
protection in the P. yoelii system (5). This is not
surprising since several studies have shown that the
protective immunity developed after immunization with
irradiated sporozoites can be independent of antibody
(3,6,7), and is dependent on CD8+ T lymphocytes (8,9).
Protective immunity after immunization with irradiated
sporozoites is also dependent on immunization with live
sporozoites suggesting that the sporozoites must enter
cells to induce the appropriate immune response. It is
not known if this immune response is directed against
antigens expressed by the sporozoite or neo-antigens
first expressed by the parasite within hepatocytes.

In an attempt to develop a vaccine that would induce a protective T cell response by presenting antigen expressed within host cells, we constructed a vaccinia recombinant that includes the entire gene encoding the P. yoelii CS protein. We now report the results of the first immunization experiments with this construct.

MATERIALS AND METHODS

Construction of the vaccinia P. yoelii CS Recombinant (Vpy).

A 1.3 Kb fragment encoding the entire P. yoelii CS protein coding region (Fig. 1) (10) was isolated by PAGE and subcloned into the Sma I site of pGS20 (11) such that the 7.5 Kb gene promoter directs transcription of the inserted DNA. Vaccinia virus recombinants were then generated as referenced previously. Briefly, monolayers of CV-1 cells were infected with wild type vaccinia virus (TK⁻ Wyeth strain) at a multiplicity of infection of 0.05 plaque forming units (pfu) per cell. Two hours post infection, recombinant plasmid DNA was introduced by transfection of calcium phosphate precipitated DNA. Cells were harvested 48 hrs later, and virus was released by 3 freeze-thaw cycles. TK⁻ recombinant virus was selected from cell lysates by plaque assay on TK⁻143 cells in the presence of 25 μg/ml 5-bromodeoxyuridine (BUdR). Isolated TK⁻ plaques were picked and used to infect fresh monolayers of TK⁻143 cells. Forty eight to 72 hrs later, when significant cytoplasmic effect (cpe) was evident, cells were harvested, subjected to 3 cycles of freezing and thawing, and cell lysates were then screened for expression of the P. yoelii CS protein by immunoblot analysis. Lysates were applied to nitrocellulose sheets with the aid of a Dot-blot manifold (Minifold I; Schleicher & Schuell, Keene NH) and unoccupied sites blocked by incubation for 1 hr in RIPA buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.5% Triton X-100) supplemented with 3% BSA (w/v). Filters were then incubated overnight at room temperature in the presence of NYS1, a monoclonal antibody specific for the P. yoelii CS protein (12) diluted 1:1000 in RIPA buffer. These were then washed 3 times with RIPA buffer, and

further incubated for 1 hr at room temperature with RIPA
containing 1 µCi ^{125}I-protein A. Filters were again
washed three times with RIPA and positive spots
visualized by autoradiography. Recombinant viruses
which were positive in this assay were subject to 3
rounds of plaque purification. Finally, plaque purified
virus was used to infect monolayers of CV-1 cells and
cell lysates were screened by western blotting to
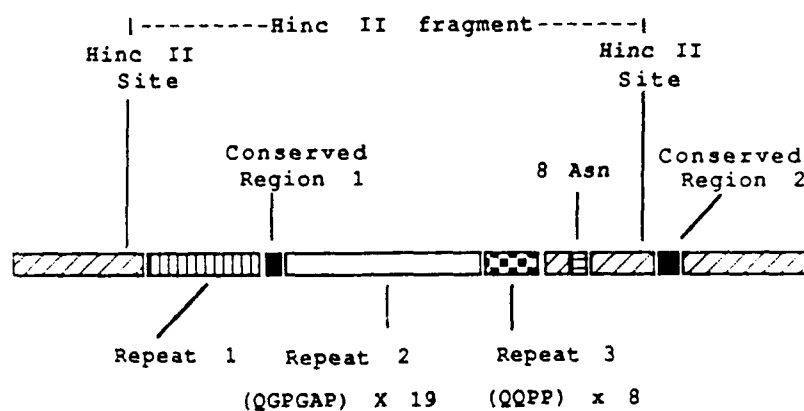confirm the presence of the full length P. yoelii CS
protein.

FIGURE 1. Diagramatic structure of the Plasmodium
yoelii gene encoding the circumsporozoite (CS) protein.

Malaria Parasites.

The 17X (NL) strain of P. yoelii yoelii was used
for all experiments. Sporozoites were isolated by
discontinuous gradient centrifugation as previously
described (13) and adjusted to required concentration
in Medium 199 supplemented with 10% mouse serum.
Irradiated sporozoites (IRRspz) used for immunization
were isolated from infected mosquitoes that had been
exposed to 10 Krads from a ^{137}Ce source.

Immunogens.

The following were using as immunogens:
a) The Vpy vaccinia CS construct encoding the full length CS protein of $\underline{P}$. $\underline{yoelii}$ (10^8 pfu/dose) inoculated intraperitoneally (ip).

b) A control vaccinia construct (Vgalk) encoding an unrelated $\underline{E}$. $\underline{coli}$ galactokinase (10^8 pfu/dose) inoculated ip.

c) IRRspz injected intravenously (iv). The first dose was 5×10^4 sporozoites, and subsequent doses were 3×10^4 sporozoites.

Immunization and Challenge Schedule.

Groups of 8 female Balb/CByJ mice (Charles River Labs), 8 weeks old, received 1, 2, 3 or 4 doses of vaccine prior to challenge. Vaccine doses were administered at 2 week intervals. Two weeks after the last immunizing dose, blood for serum was obtained and the mice were challenged with infective sporozoites. Mice that received 1, 2 and 3 doses were challenged with 10^4 sporozoites, while mice that received 4 doses of vaccine were challenged with 200 sporozoites. Mice were then monitored for parasitemia as an indicator of protective immunity.

Detection of Antibodies to Sporozoite Epitopes.

Pooled sera were analyzed for reactivity with sporozoite antigens using the following techniques:
Indirect fluorescent antibody test (IFAT). The IFAT was carried out using air dried whole sporozoites as previously described (12).
Enzyme linked immunosorbent assay (ELISA). ELISA was used to analyze the epitopes on the CS protein recognized by sera from the different groups of vaccinated mice. Briefly, the test consisted of a solid phase assay in which synthetic peptides or recombinant proteins were coated to the wells of 96 well flat bottom Immulon II microtiter plates (Dynatech Laboratories, Alexandria,VA). Wells were coated with one of the following: a) a recombinant fusion protein produced by the Hinc II fragment CS gene in $\underline{E}$. $\underline{coli}$ that includes the 3 $\underline{P}$. $\underline{yoelii}$ repeat domains and the conserved Region I sequence fused to 81 amino acids from the

non-structural protein of viral influenza;   b) a
synthetic peptide containing 2 copies of repeat #2,
Gln.Gly.Pro.Gly.Ala.Pro [QGPGAP]$_2$; and c) a synthetic
peptide containing 3 copies of repeat #3,
Cln.Gln.Pro.Pro [QQPP]$_3$. Each well was then blocked
with casein and incubated overnight. The wells were
then reacted with the appropriate dilution of a test
serum, washed 3x with buffer after which the revealing
antibody, a horseradish peroxidase (HRP) labelled goat
anti-mouse Ig was added followed by the peroxidase
substrate consisting of equal volumes of H$_2$O$_2$ and ABTS
(2.2'-azino-di[3-ethyl- benzothiazoline sulfonate])
(Kirkegaard and Perry Laboratories, Inc., Gaithersburg
MD). Color development was measured photo-metrically at
405 nm using Microelisa Autoreader MR 580 (Dynatech
Laboratories).

Depletion of CD8+ T Lymphocytes.

    Mice immunized with 4 doses of irradiated
sporozoites and shown to be protected against challenge
with P.yoelii sporozoites 17 days previously were given
two 1 mg ip injections of monoclonal antibody 19/178 at
24 hour intervals to deplete them of CD8+ T lymphocytes
as previously described (8). They were challenged with
either 200 or 10,000 sporozoites 48 hours later.
Treatment with Mab 19/178 was continued every third day
after challenge until blood parasites appeared. Blood
films were examined for parasites until patency or for
14 days, whichever occurred sooner. When parasites were
detected in the blood, mice were killed and spleen cells
prepared and analysed for depletion of the subpopulation
carrying the CD8 marker by fluorescent activated cell
sorter. (FACS).

RESULTS

Protection Against Challenge.

    Results of the challenge of mice immunized with Vpy
are summarized in Table 1. Forty percent of the mice
that received a single dose of irradiated sporozoites
were protected against challenge with 10^4 sporozoites,
and this level of protection increased to 100% in the

three groups of mice that received 2, 3, or 4 doses of
irradiated sporozoites. In contrast, all mice immunized
with the recombinant Vpy construct became infected after
challenge including the group that had received 4 doses
of the construct and was challenged with only 200
sporozoites.

TABLE 1

Results of challenging mice immunized with vaccine
preparations Vpy, Vgalk and IRRspz.

Expt	No.of Doses	Vaccine Preparation	IFAT Titer	Ratio of Mice Prot./Chall.
Challenged with 10^4 sporozoites/mouse.				
A	1	Vpy	32	0/8
		Vgalk	<8	0/8
		IRRspz	16	3/8
		Control	<8	0/8
B	2	Vpy	512	0/8
		Vgalk	<8	0/8
		IRRspz	256	8/8
		Control	<8	0/8
C	3	Vpy	2,048	0/8
		Vgalk	<8	0/8
		IRRspz	2,048	8/8
		Control	<8	0/8
Challenged with 200 sporozoites/mouse.				
D	4	Vpy	1,024	0/8
		Vgalk	<8	0/8
		IRRspz	2,048	3/6
		Control	<8	0/8

Antibody levels.

Anti-sporozoite antibody titers as measured by IFAT
were comparable in the groups that were vaccinated with
IRRspz and those that received the Vpy construct.
Results obtained by ELISA using the Hinc II fusion
protein containing all the repeat domains were also
consistent with the IFAT findings. However, although
both groups had made comparable levels of antibodies to
Hinc II peptide (Fig.2), there was a significant
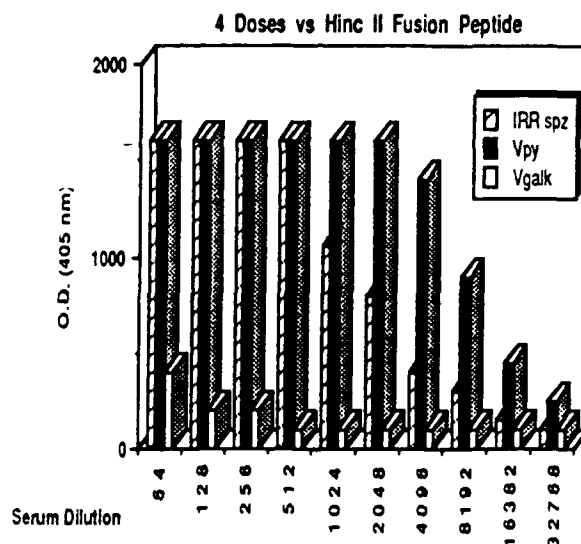difference in the epitopes recognized by each at the
endpoint titer.

FIGURE 2. ELISA determination of the antibody
response to the Hinc II fusion peptide of mice immunized
with vaccine preparations IRRspz, Vpy and Vgalk.

Mice immunized with irradiated sporozoites produced
significantly higher levels of antibodies to repeat #2
([QGPGAP]$_2$) while mice immunized with the Vpy construct
made higher levels of antibodies to repeat #3 ([QQPP]$_2$)
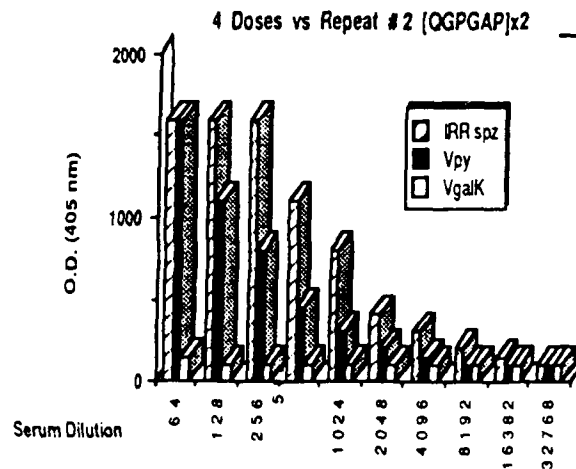(Figs.3 and 4 respectively).

4 Doses vs Repeat # 2 [QGPGAP]x2

FIGURE 3. ELISA determination of the antibody
response to Repeat #2 [QGPGAP]$_2$ of mice immunized with
vaccine preparations IRRspz, Vpy and Vgalk.
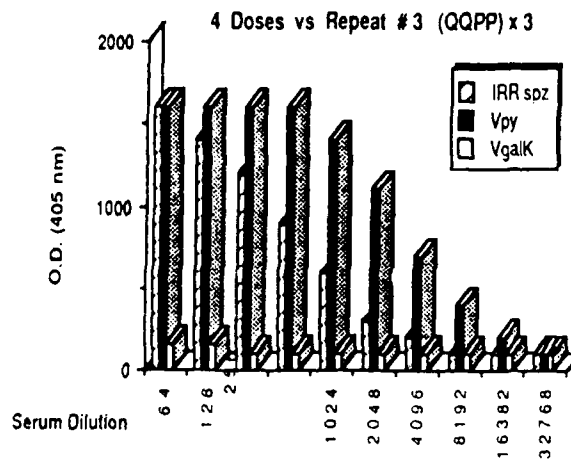
4 Doses vs Repeat # 3 (QQPP) x 3

FIGURE 4. ELISA determination of the antibody
response to Repeat #3 (QQPP)$_3$ of mice immunized with
vaccine preparations IRRspz, Vpy and Vgalk.

Protection After CD8 T Cell Depletion.

We knew that a Mab to repeat #2 protected mice in passive transfer experiments, whereas a second Mab reacting with repeat #3 did not (Charoenvit, in preparation) and we wondered if the difference in antibody specificity between the IRRspz and Vpy immunized groups might account for the differences in protection observed in each, especially for low challenge doses.

TABLE 2
Results of rechallenging protected mice
depleted of   CD8+ T-lymphocytes

Mouse No.	IFAT Titer at challenge	Day after Challenge Blood Infection Detected		
		+3	+4	+5
Challenged with 200 sporozoites/mouse.				
CD8+ T cell depleted.				
1	512	–	–	–
2	1,024	–	–	–
3	512	–	–	+
4	2,048	–	–	–
Challenge controls.				
1	<8	–	+	
2	<8	–	+	
3	<8	–	+	
4	<8	–	+	
Challenged with 10,000 sporozoites/mouse.				
CD8+ T cell depleted.				
5	1,024	–	–	
6	2,048	+		
7	512	+		
8	1,024	+		
Challenge controls.				
5	<8	+		
6	<8	+		
7	<8	+		
8	<8	+		

However, when mice immunized with 4 doses of irradiated
sporozoites were depleted of CD8+ T cells (verified by
FACS analysis; data not shown) and challenged with
either 200 or 10^4 sporozoites, they all became infected
indicating that the antibodies induced by immunization
with IRRspz were not sufficient to protect these mic
against challenge with 10^4 sporozoites or even the very
low challenge dose of 200 (Table 2).

DISCUSSION

Observations in the rodent malaria model systems
have provided the foundation for development of human
malaria sporozoite vaccines.   Murine studies now
clearly demonstrate the importance of cell-mediated
immune mechanisms in the protective immunity elicited by
immunization with irradiated sporozoites (3,7,8,9).   In
an attempt to induce a protective cellular immune
response directed against the CS protein, we constructed
a P. yoelii-CS vaccinia recombinant.   The failure of
this vaccine to protect against even a minimal
sporozoite challenge indicates that either the CS
protein is not the target of such immunity, or that
intraperitoneal immunization with Vpy did not induce the
required cellular immune response.   Recent studies in
the P. berghei system (Sadoff J; personal communication)
indicate that the CS protein may be the target of such
an immune response.   If so, this vaccinia recombinant
may be an inadequate vector for properly delivering the
immunogen, or the route of administration may have been
inappropriate for induction of the cytotoxic T cells
that we believe are involved in a protective response.
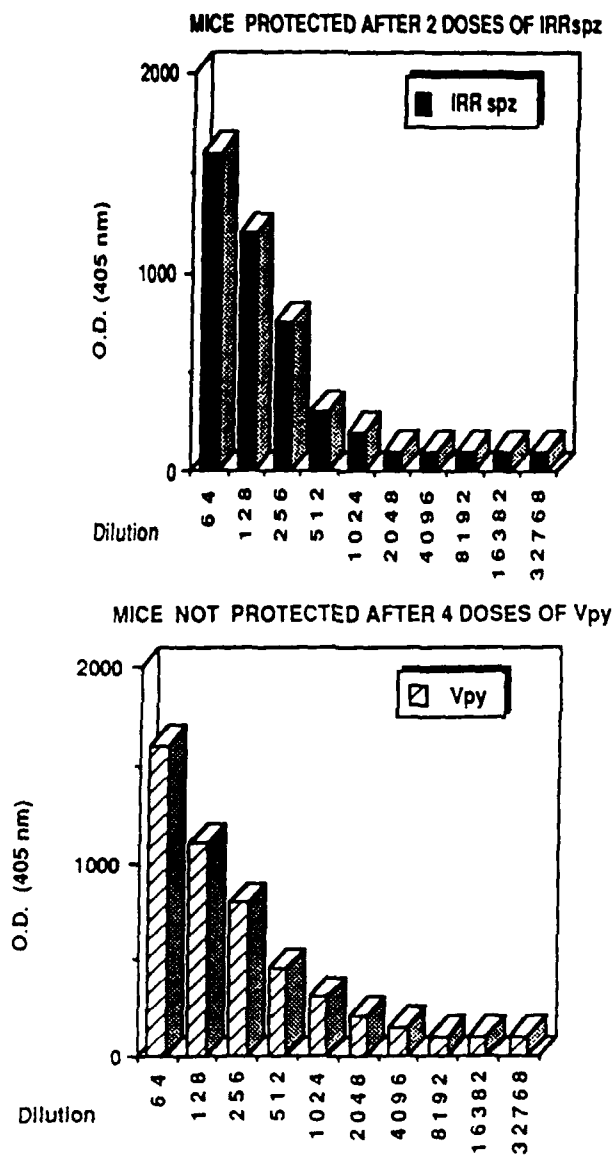Further studies are in progress to explore these
possibilities.

MICE PROTECTED AFTER 2 DOSES OF IRRspz

MICE NOT PROTECTED AFTER 4 DOSES OF Vpy

FIGURE 5. Comparison of the ELISA response to
Repeat #2 [QGPGAP]$_2$ in mice vaccinated with the IRRspz
and Vpy vaccine preparations. The IRRspz group was
completely protected after 2 vaccine doses, whereas the
Vpy group was totally suscpetible after 4 doses of Vpy.

Mice immunized with 4 doses of Vpy were not protected against challenge with even 200 sporozoites, although they had levels of antibody against CS protein and sporozoite epitopes comparable to the levels in mice immunized with 2 doses of IRRspz that were protected against challenge with 10,000 sporozoites (Fig.5).

The results of the CD8+ depletion study (Table 2) demonstrate that circulating antibody levels in mice immunized with 4 doses of irradiated sporozoites are not adequate to protect against even a low dose challenge. Protective immunity in IRRspz immunized mice appears to be wholly dependent on CD8+ T cells regardless of the size of challenge.

Although IRRspz immunized mice are not protected by antibodies, we have shown that passive transfer of a monoclonal antibody, NYS1 directed against repeat #2 (QGPGAP) can protect against challenge with a large inoculum of sporozoites (Charoenvit,in preparation). It is therefore theoretically possible that vaccines eliciting antibodies against the CS antigen will still prove protective, if the levels of antibody they achieve exceed those reached with the IRRspz and Vpy preparations used in this study. Our data provide the first indication that the immunodominance of B cell epitopes on the CS protein may be dependent on how the immunogen is delivered. Antibodies measured by IFAT were similar in the IRRspz and Vpy immunized groups. However, immunization with the IRRspz vaccine induced the highest levels of antibodies against [QGPGAP] in contrast to immunization with Vpy which produced higher levels to [QQPP]. The protective Mab, NYS1, is directed against an epitope in the [QGPGAP] domain, whereas a second Mab, NYS3 reacting with a different epitope contained in the [QQPP] domain is not protective (Charoenvit, in preparation). It would appear that future studies of subunit vaccines in this model system would be well advised to consider the epitope specificity of vaccine induced antibodies and not rely on antibodies to the entire sporozoite as the sole measure of immunogenicity.

ACKNOWLEDGMENTS

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